High level expression and purification of active recombinant human interleukin-15 in *Pichia pastoris*

Wei Sun[a,b], Yunxin Lai[a], Hongbo Li[c], Tao Nie[a], Ye Kuang[a,d], Xiaofeng Tang[a], Kuai Li[a], P. Rod Dunbar[e], Aimin Xu[a,f], Peng Li[a*] and Donghai Wu[a*]

[a]The Key Laboratory of Regenerative Biology, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences and Guangdong Provincial Key Laboratory of Stem Cell and Regenerative Medicine, Guangzhou, 510530 China;

[b]School of life sciences, Anhui University, Hefei 230601, China;

[c]Key Laboratory of Research and Utilization of Ethnomedicinal Plant Resources of Hunan Province, Department of Life Sciences, Huaihua College; Huaihua, China;

[d]Department of Biomedical Engineering, School of Pharmaceutical Sciences, Jilin University, Changchun, China;

[e]School of Biological Sciences & Maurice Wilkins Centre, University of Auckland, Auckland, New Zealand.

[f]Department of Pharmacology and Medicine, the University of Hong Kong, Hong Kong, China.

[*]**Corresponding authors:** Donghai Wu or Peng Li,
Tel: +86-20-32015205, Fax: +86-20-32015299 E-mail: wu_donghai@gibh.ac.cn or li_peng@gibh.ac.cn.

Abstract

Interleukin-15 (IL-15) is a pleiotropic cytokine and a member of the four α-helix bundle family of cytokines which include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. IL-15 exhibits a broad biological activity and induces the differentiation and proliferation of T, B and natural killer (NK) cells. In this study, a DNA fragment containing the mature human IL-15 sequence was cloned into pPICZaA vector, generating a fusion protein with the alpha factor signal sequence in the N-terminus and 6×His as well as c-Myc tags in the C-terminus. The resulting plasmid was integrated into the genome of *Pichia pastoris* strain X-33. Recombinant yeast transformants with high-level recombinant human IL-15 (rhIL-15) production were identified, which secrete as much as 75 mg/L rhIL-15 after 3 days of induction by methanol. The rhIL-15 was purified by Ni^{+}-NTA affinity chromatography, followed by DEAE anion exchange, yielding over 95% highly purified rhIL-15. Mass spectrometry and MALDI-TOF-TOF analysis showed the purified rhIL-15 had larger molecular weights than expected, due to different degrees of N-linked glycosylation. The biological activity of the rhIL-15 proteins was measured by its ability to enhance cellular proliferation of CTLL-2 and NK cells. The results demonstrate that the experimental procedure we have reported here can produce a large amount of active recombinant human IL-15 from *Pichia pastoris*.

Key words： Interleukin-15, *Pichia pastoris*, Expression and purification,N-linked glycosylation

1. Introduction

Interleukin - 15 (IL-15) was discovered in 1994 by two different laboratories, and characterized as T cell growth factor [1, 2]. IL-15 has been shown to stimulate the proliferation of activated T cells as well as to facilitate the induction of cytotoxic T-lymphocytes, and the generation, proliferation and activation of NK cells [3-8]. As a potent pro-inflammatory cytokine, IL-15 plays an important and complex role in autoimmune disease and inflammation [9, 10]. Previous studies have indicated that IL-15 is an effective vaccine adjuvant when administered as a plasmid DNA or a recombinant protein in combination with DNA vaccines against infectious pathogens [11-13]. In addition, IL-15 plays a pivotal role in some hematological malignancies and presents anti-tumor effects [14-16]. The direct administration of IL-15 has shown anti-tumor effects in several preclinical mouse tumor models [17, 18].

In contrast to the well-defined biological activities of IL-15, methodologies to generate fully-functional IL-15 protein in large quantities at low cost have barely been reported. Although commercial recombinant human IL-15 is available, the limited biological activity and high price have largely hampered its applications in both basic research and the clinic. Methods for the production and purification of rhIL-15 from *Escherichia coli* and Chinese hamster ovary (CHO) cell have previously been attempted and reported [19, 20]. However, there are no reports of its expression in *P. pastoris*, a system that has become popular in recent years due to its low cost, ease of genetic manipulation, capacity to grow to a high cell density, and lack of pyrogenic endotoxins [21]. Many pharmaceutically important proteins have been successfully produced using this system for clinical applications [22, 23]. In this study, we expressed rhIL-15 using the yeast *P. pastoris* and report our establishment of a highly efficient expression system for biologically active human recombinant IL-15 in *P. pastoris* with the potential for large-scale production in bioreactors.

2. Materials and methods

2.1. Materials

P. pastoris strain X-33, expression vector pPICZaA, Yeast nitrogen base (YNB), D-sorbitol, D-biotin, and BCA Protein Assay kit were purchased from Pierce (Guangzhou, China). Plasmid extraction kit, restriction enzymes, DNA polymerase and T4 DNA ligase were purchased from Takara (Guangzhou, China). Primers 5’ Factor and 3’ Oxidase I was synthesized by Invitrogen (Shanghai, China). Escherichia coli transformants were selected on LB agar plates containing low salt (1% peptone, 0.5% NaCl, 0.5% yeast extract, and 1.5% agar) and 25 mg/L zeocin. *P. pastoris* transformants were initially selected on YPDZ plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) with 100 mg/L zeocin, then on YPDZ plates containing increasing concentrations of zeocin (from 500, 1000, 2000 to 3000 mg/L, respectively) for the isolation of high copy number recombinants. P. pastoris was grown in BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen broth, 0.4 mg/L biotin, 1%

glycerol), and induced in BMMY medium which is the same as BMGY except that glycerol was replaced by methanol. Dialysis bags with molecular weight cut-off (MWCO) of 5 KDa were purchased form Millipore (Guangzhou, China). AKTA FPLC、Ni+-NTA columns (HisTrapTM HP,5ml), DEAE-Sepharose FF ion exchange columns (Hi Trap™, 1 ml) were purchased from GE Healthcare (Guangzhou, China). PNGase F was purchased from NEB (Guangzhou, China). Commercial recombinant human IL-15 proteins were purchased from PEPROTECH (Guangzhou, China). Mouse CTLL-2 cells were purchased from ATCC (Beijing, China). Cord blood samples were collected at Department of Gynecology and Obstetrics, the South China Medical University (SCMU) (Guangzhou, China) for research purpose only , and this process was monitored by the Institutional Review Boards of SCMU. Usage of NOD/SCID/IL2Rg-/- (NSI) mice was approved by the Institutional Animal Care and Use Committee (IACUC) of the Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences.

2.2. Plasmid construction

Human IL-15 cDNA (399bp) was amplified by PCR from a plasmid encoding full-length human IL-15 cDNA (Genscript, Nanjing, China). An EcoRI site was introduced to allow in-frame cloning behind a-factor secretion signal of pPICZaA and a nucleotide sequence encoding the KEX2 cleavage site was placed upstream of the IL-15. Forward and reverse primers used were 5'-GAATTCGGCATTCATGTCTTCATTTTG-3' and 5'-GCGGCCGCAGAAGTGTTGA TGAACATTTG-3', respectively. The PCR products were digested with EcoRI and NotI, and the digested fragment was inserted between the EcoRI and NotI sites of pPICZaA where the IL-15 is under the control of the alcohol oxidase1 promoter on the vector. The resulting construct also contains a c-Myc tag as well as a 6×His-tag at C-terminus.

2.3. Transformation of *P. pastoris* and selection of transformants

The expression vector was linearized by digestion with SacI and transformed into *P. pastoris* X-33 using Lithium Chloride transformation method as recommended (Invitrogen, 2008). P. pastoris transformants were grown on YPDZ plates containing 100 μg/ml of zeocin. Resulting colonies were transferred to YPD plates containing 500, 1000, 2000 and 3000 μg/ml of zeocin, respectively. Colonies that grew on YPD plates containing 3000 μg/ml of zeocin were picked and subjected to PCR amplification using the primers 5' factor and 3' Oxidase I. The correct transformants were selected for further studies.

2.4. Small-scale fermentation and time course expression study

Selected colonies from the YPD plate containing 3000 μg/ml of zeocin were grown in 100 ml BMGY growth medium in 1000 ml flasks with constant shaking (250 rpm) at 30℃ for 18 h until the OD600 measured between 6.0 and 8.0. The cultures were pelleted by centrifugation for 5 min at 2000 g. For the induction phase, the cell pellets were inoculated into 20 ml of BMMY induction media in 250 ml baffled flasks and grown for 120 h at 28℃ with constant shaking at 250 rpm, as recommended by the manufacturer (Invitrogen, 2008). Each day, 0.2 ml of 100% methanol was added into the culture (final 1.0%) to induce the protein expression. 1 ml cell cultures were collected at 0 h, 24 h, 48 h, 72 h, 96 h and 120 h after the addition of methanol. Protein concentrations of the collected supernatants were assayed with a Bradford protein assay kit. 0.1 ml of these eluted solution were mixed with 0.5 ml of ice cold acetone. After centrifugation, precipitates were re-suspended in 40 μl of 1×SDS loading buffer and heated at

95 ℃ for 10 min and the denatured samples were subjected to 15% SDS-PAGE analysis.

2.5. Large-scale fermentation and purification

Scale-up expression was carried out in a 5 L baffled flask. *P. pastoris* harboring pPICZaA/hIL-15 with resistance to 3000 μg/ml of zeocin was grown in 1 L BMGY medium by constant shaking (250 rpm) at 30 ℃ till OD600 = 6.0-8.0. Cells were harvested and resuspended in a 1 L baffled flask containing 0.2 L BMMY medium, then cultured for 96 h. Methanol was added to the medium to a final concentration of 1.0% every 24 h. After the 96 h induction, all of the cell cultures were centrifuged for 15 min at 2000 g. Protein concentration in the supernatant was estimated by Bradford assays, and the supernatant was collected and dialyzed against 2 L buffer A (50 mM Tris-HCl, 200 mM NaCl, 20 mM imidazole pH 8.0) at 4 ℃ overnight. After centrifugation at 20,000g for 30 min at 4 ℃, the supernatants were loaded onto a 5 ml Ni^{+}-NTA column which was pre-equilibrated using buffer A. Then the column was washed with 60ml buffer A (50 mM Tris-HCl, 200 mM NaCl, 20 mM imidazole pH 8.0) to remove unbound proteins. The recombinant IL-15 was eluted with a linear gradient of 0-0.5 M imidazole buffer B (50 mM Tris-HCl, 200 mM NaCl, 500mM imidazole pH 8.0). 0.1 ml of these eluted solution were mixed with 0.5 ml of ice cold acetone. After centrifugation, precipitates were resuspended in 40 μl of 1xSDS loading buffer. The samples were subjected to SDS-PAGE (15% gel) analyze and stained with Coomassie blue R250.

2.6. Deglycosylation analysis of rhIL-15

The IL-15 recombinant proteins were eluted from a Ni+-NTA column and dialyzed in a dialysis bag against 2 L ice-cold dialysis buffer I (20 mM Tris-HCl, pH 8.0) overnight. After centrifugation at 20,000 g for 30 min at 4 ℃, the dialyzed IL-15 solution was filtered through a 0.22 μm filter and loaded onto a DEAE-Sepharose FF ion-exchange column that was pre-equilibrated with 50 ml buffer I using an AKTA FPLC system. After loading, the column was washed with 100 ml buffer I and eluted with a linear gradient of 0-0.5 M NaCl in buffer I. Fractions containing the proteins were collected and assayed by 15% SDS–PAGE. 50 μl purified rhIL-15 protein from the DEAE ion-exchange column was deglycosylated with PNGase F and then analyzed by 15% SDS-PAGE.

2.7. Matrix assisted laser desorption ionization-mass spectroscopy

Two main protein bands from Coomassie stained SDS-PAGE gel of deglycosylated rhIL-15 were cut and washed twice with 50 mM NH4OAc and 30% acetonitrile (ACN) (40μl) for 30 min for destaining. The gels were dehydrated with 40 μl of ACN for 3 min and completely dried under N2 for 20 min. The gels were digested with 50 μg/ml trypsin in digestion buffer (50 mM NH4OAc and 0.5 mM CaCl2) for 1 h at room temperature, followed by addition of 40 μl of digestion buffer (final pH 7.0) and incubated at 37 ℃ for 16-20 h in a shaker bath. After digestion, samples were centrifuged at 10,000 rpm for 20 s, supernatants were collected, and the gels were extracted again with solvents at 30 ℃ for 30 min in a shaker bath in the following order: 0.1% TFA (40 μl), 30% ACN and 0.1% TFA (aq, 40μl), and 60% ACN and 0.1% TFA (aq, 40 μl). After each extraction, samples were centrifuged at 10,000 rpm for 20 s and all supernatants were combined, including the initial supernatant (pH 4.0), dried under N2, and stored at -80 ℃ until MS analysis, using PerSeptive Biosystem's Voyager-DE MALDI TOF mass spectrometer (Framingham, MA/Applied Biosystems, Foster City, California, USA) equipped with a 337 nm nitrogen laser. Peptides from mass spectra of in-gel digest samples were

matched against databases of Swiss-Prot, NCBInr, and MSDB using the Mascot search engine (Matrix Sciences) for PMF.

2.8. Cell proliferation assays

2.8.1. CTLL-2 cell proliferation assay

CTLL-2 cells (ATCC) were washed three times in 10 ml fresh RPMI 1640 medium and centrifuged at 1000g for 5 min. Cells were resuspended in RPMI 1640 medium containing 10% FBS. 2×10^4 CTLL-2 cells were added into each well of a 96-well plate. Various concentrations of rhIL-15 (0, 0.01, 0.05, 0.25, 2.5, 25 ng/ml) were added to each well. The plates were then incubated under standard conditions (37℃and 5% CO2) for 48 h and cell proliferation was determined using Cell Titer-Glo (Promega, Guangzhou, China) according to the manufacturer's instructions. Luminescence was read in a Wallac Victor absorbance/luminescence reader, and data analyzed using GraphPad Prism software using four-parameter logistic curve fitting.

2.8.2. Natural kill cell proliferation assay in vitro

All primary samples were obtained with informed consent for research purposes, and the procedures were approved by the Research Ethics Board of Guangzhou Institutes of Biomedicine and Health (GIBH). Mononuclear cells in human cord blood were separated by Ficoll-Hypaque density centrifugation (800 g, 20 min) and the NK cells were collected from mononuclear cells by Magnetic-activated cell sorting (MACS). NK cells were washed three times in 10 ml fresh RPMI 1640 medium and centrifuged at 1000g for 5 min. Cells were re-suspended in RPMI 1640 medium containing 10% FBS and 1×10^6 cells were added into each well of a 24-well plate. RhIL-15 (50 ng/ml) was added to the plate wells and the same volume of PBS was used as negative control. The plates were then incubated under standard conditions (37℃ and 5% CO_2). Cells were collected at day 4 and day 8, stained with APC-CD56 and FITC-CD3, and analyzed on a Beckman-Coulter FC500 flow cytometer for the proportion of the cultures comprised of live NK cells.

2.8.3. Natural kill cell proliferation assay *in vivo*

NSI mice derived at GIBH have been described previously[24]. Animal experiments were performed in the Laboratory Animal Center of GIBH, and all animal procedures were approved by the Animal Welfare Committee of GIBH. All mice were maintained in specific-pathogen-free cages and provided with autoclaved food and water. Human NK cells were washed three times in 10 ml fresh PBS, then 3×10^6 human NK cells were injected into NOD/SCID/IL-2Rg-/- mouse under the ophthalmic vein. The NK cell-treated SCID mice were divided into two groups; one group was treated daily with 200 μg rhIL-15 for 4 days while the other group was treated with PBS. Mouse peripheral blood, spleen and bone marrow were collected at day 5, lysed for red cells and stained with hCD45-PE and hCD56-APC antibodies to detect human NK cells, before analysis on a Beckman-Coulter FC500 flow cytometer.

3. Results

3.1. Construction of rhIL-15 expression vector and transformation of *P. pastoris*

Using a custom designed primer pair, the desired fragment of the human IL-15 gene was

amplified by PCR (see Materials and Methods). The IL-15 gene was cloned into pPICZaA, where it is under the control of Oxidase I promoter on the vector (Fig. 1a). The recombinant human interleukin-15 nucleotide sequence is as follows: 5'-GCTCCCATGACCCAGACAACG
TCCTTGAAGACAAGCTGGGTCAACTGCTCTAACATGATCGATGAAATTACAACACAC
TTAAAGCAGCCACCTTTGCCTTTGCTGGACTTCAACAACCTCAATGGGGAAGACCAA
GACATTCTGATGGAAAATAACCTTCGAAGGCCAAACCTGGAGGCATTCAACAGGGC
TGÆGCTCCCATGACCCAGACAACGTCCTTGAAGACAAGCTGGGTCAACTGCTCTAA
CATGATCGATGAAATTACAACACACTTAAAGCAGCCACCTGCTCCCATGACCCAGA
CAACGTCCTTGAAGACAAGCTGGGTCAACTGCTCTAACATGATCGATGAAATTACAA
CACACTTAAAGCAGCCACCTTTGCCTTTGCTGGACTTCAGAACAAAAACTCATCTCA
GAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATTGA-3' (contains a c-Myc tag and a 6×His-tag at the C-terminus). The resulting plasmid, pPICZaA/hIL-15, was further confirmed by sequencing before transformed into *P. pastoris* by Lithium Chloride transformation method and plated on YPDS medium containing low zeocin (100 mg/L). After selection through higher levels of zeocin, three colonies grown on YPDZ (3000 mg/L zeocin) were picked and subjected to PCR amplification using the primer 5' Factor and 3' Oxidase I. As shown in Fig. 1b, the amplified fragment was about 750 bp as expected, indicating that the pPICZaA/hIL-15 plasmid was successfully introduced into the *P. pastoris* genome.

3.2. Small-scale fermentation and time course study

A single proven pPICZaA/hIL-15 colony of *P. pastoris* was grown in 100 ml of BMGY and induced with methanol in 20 ml BMMY for 5 days, and supernatant samples were collected at 0 h, 24 h, 48 h, 72 h, 96 h and 120 h. As shown in Fig. 2A, an about 17 KDa secreted protein band was detected by Coomassie blue R250 stain at 48 h post induction with methanol and gradually reached a maximal level after 4 days of induction, and the band decreased thereafter with substantial degradation, probably by yeast proteases secreted into the culture medium (Fig. 2). The size of observed recombinant protein is similar to the estimated molecular weight (MW) of rhIL-15 (the theoretical MW of 17.8 KDa, pre-determined using Expasy Compute PI/Mw tool, http://www.expasy.ch/cgi-bin/pi_tool) based on the predicted amino acid sequence of:
GIHVFILGCFSAGLPKTEANWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMK
CFLLELQVISLESGDASIHDTVENLIILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSF
VHIVQMFINTSLAAASFLEQKLISEEDLNSAVDHHHHHH (c-Myc tag and 6×His-tag as indicated). Because mature native human IL-15 protein is a 15-17 KDa glycoprotein containing 133 amino acids with two potential N-linked glycosylation sites (NXS: N119NS and N127VT), it is likely that three proteins (about 17 KDa, 22 KDa and 55KDa) are rhIL-15 proteins, representing different degrees of N-glycosylation.

3.3. Large-scale fermentation and purification with Ni^{+}-NTA column

After 3 days of induction with 1% methanol, all the 0.2 L culture medium was centrifuged and the supernatant was collected. The recombinant IL-15 was eluted using a linear gradient of 0-0.5 M imidazole with buffer B (50 mM Tris-HCl, 200 mM NaCl, 500 mM imidazole pH 8.0), and fractions were collected (Fig.3a). The purified recombinant protein was recovered and analyzed by SDS-PAGE and three major protein bands with molecular weights of 17 KDa and 22 KDa and 55 KDa were observed (Fig. 3b). Approximately 15 mg protein was obtained from 200 ml of crude culture supernatant through purification with Ni^{+}-NTA column. As mature native human IL-15 has two disulfide bonds (positions C83-C133、C90-C136) it may exist as a

polyprotein state at native condition. The result of SDS-PAGE conducted at native and denatured condition confirmed this state (Figure. 3c).

3.4. Deglycosylation analysis of rhIL-15

After dialysis, the rhIL-15 was further purified using DEAE chromatography which eliminated most of the contaminating proteins. It is well known that *P. pastoris* can attach N-linked glycans to secreted proteins. PNGase F is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins. The purified proteins were deglycosylated by PNGase F and the SDS-PAGE results showed that the rhIL-15 protein with molecular mass of about 22 KDa and 55KDa was indeed an N-glycosylated protein (Fig. 4).

3.5. Mass spectrometric analysis

The two main protein bands were cut out from the deglycosylated rhIL-15 gel and further analyzed by MALDI-TOF-TOF mass spectrometry following trypsin digestion. Mass spectrometric analysis based on searching the vertebra database confirmed the two bands with different molecular mass were all rhIL-15 protein (Fig. 5a-5b).

3.6. Biological activity of rhIL-15:

3.6.1. CTLL-2 cell proliferation assay

A CTLL-2 cell proliferation assay was conducted to determine the specific biological activity of the purified rhIL-15. As shown in Fig. 6, CTLL-2 was unable to proliferate in the absence of rhIL-15 while addition of as little as 0.01 ng/ml of rhIL-15 stimulated the growth of CTLL-2 cells. A cell proliferation assay therefore demonstrated the potent biological activities of the rhIL-15 purified from *P. pastoris*.

3.6.2. NK cell proliferation assay

CTLL-2 is a transformed murine cell line and its response may not reflect the activity of soluble IL-15 on lymphocytes in the blood of humans or other primate species. We therefore generated separate IL-15 dependent assays that measured the expansion of NK cells. In an in vitro assay, the proportion of live cells that were $CD56^{+}$ $CD3^{-}$ NK cells at day 4 was 67.6% in wells exposed to rhIL-15, higher than the proportion of live NK cells of wells exposed to PBS (43.7%). At day 8 this difference was more striking, with the proportion of live cells that were NK cells being 71.5% in wells exposed to rhIL-15 was and only 16.8% in the PBS controls (Figure 7a). In an in vivo assay using a humanized mouse model, 3×10^{6} human NK cells were injected into each mouse and mice were treated intraperitoneally with 200ug IL-15 or 200ul PBS (n=3) once per day for 4 days, and on day 7, mice were sacrificed to detect NK cells in the peripheral blood, spleen and bone marrow. The proportion of live cells in peripheral blood, spleen and bone marrow that were NK cells were much higher in animals injected with rhIL-15 than in those injected with PBS (5.08%, 5.64% and 0.028% respectively for rhIL-15, vs 0.00%, 0.003%, and 0.00% for PBS, as shown in Figure 7b). These data confirm that purified rhIL-15 from P. pastoris could sustain human NK cells both in intro and in vivo.

4. Discussion

The methylotrophic yeast *P.pastoris* is a single-celled microorganism that is easy to

manipulate and culture. It is also an eukaryote and capable of many of the same post-translational modifications as higher eukaryotic cells such as proteolytic processing, disulfide bond formation and glycosylation [25]. The *P. pastoris* system is also generally regarded as being faster, easier, and less expensive than the other systems. Thus, *P.pastoris* is widely used for the production of recombinant proteins[21]. Previously, IL-15 was produced using an E. coli expression system with a low recovery of soluble protein. To our knowledge, this is the first report of highly efficient production and purification of active rhIL-15 (75 mg/L) using *P. pastoris.*

The yield of recombinant proteins is affected by a variety of factors at both the genetic and cultural levels [25], so high level secreted yields cannot be assumed using standard protocol provided by the vendor. Previously we found that optimization of Kex2 P1' site residue could largely increase the *P. pastoris* secretory productivity of recombinant proteins[27]. In this current study, we could not obtain high level expression of rhIL-15 initially using standard protocols (data not shown). So we try to modify the rhIL-15 express vector by replace the Kex2 P1' alanine with proline, after optimizing the Kex2 P1' site residue we ultimately achieved high level expression of rhIL-15 in *P. pastoris*. Therefore, optimization of Kex2 P1' site residue was one critical factor determining the level of rhIL-15 expression in *P. pastoris.*

Although *P.pastoris* has the capacity of performing many of the posttranslational modifications as mammalian cells, N-glycosylation is different from the mammalian systems[28]. The rhIL-15 obtained from *P. pastoris* was larger than the predicted molecular mass based on the amino acid sequence (17.8 KDa), suggesting the various recombinant hIL-15 protein molecules purified were glycosylated to different degrees. PNGase F deglycosylation analysis confirmed the rhIL-15 proteins with molecular mass of about 22 KDa and 55 KDa were N-glycosylated proteins.

Interleukin-15 exerts powerful stimulatory effects on lymphocyte subsets that result in antiviral and anti-tumoral activities. Our study confirmed that glycosylated rhIL-15 from *P. pastoris* could stimulate mouse CTLL-2 cells and the proliferation of human NK cells both in vitro and in vivo. Recombinant human IL-15 produced from *P. pastoris* would be a useful tool to set up humanized mouse model or be further exploited as an antiviral and anti-tumoral medicine as well as a vaccine adjuvant.

In summary, though optimizing the Kex2 P1' site residue, we have optimized conditions that allow successful production of functional recombinant human IL-15 in *P.pastoris* for the first time to the best of our knowledge. A large quantity of highly-purified active rhIL-15 would be an invaluable tool to investigate its structural and biochemical properties as well as to facilitate its clinic applications.

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Figure legends:

Fig.1 (a) Schematic illustration of expression vector of pPICZaA/hIL-15. (b) PCR results of rhIL-15 *P. pastoris* transformants. One colony of untransformed X-33 *P. pastoris* and three colonies of rhIL-15 *P. pastoris* transformants from YPDZ plate (2000 marker; lane 1-3, transformants of pPICZaA/hIL-15; lane #, an untransformed colony as negative control).

Fig.2 Time course of the rhIL-15 expression in baffled flask. Culture supernatants were collected at the indicated time after methanol induction. The supernatants were precipitated with acetone, analyzed on 15% SDS–PAGE and stained with Coomassie blue R250. Recombinant hIL-15 protein is indicated with an arrow.

Fig.3 Purification of rhIL-15 expressed in *P. pastoris*. (a) Elution curves of rhIL-15 from Ni^{+}-NTA chromatography column in buffer B. The eluents were collected at the value of OD280>200 mAU, totally collected 15 mL. (b) Recombinant hIL-15 was purified by Ni^{+}-NTA column, separated on SDS-PAGE and stained with Coomassie blue R250. Lane 1#, Culture supernatants after 72 h methanol induction; lane 2#, unbound protein in 72 h methanol induction supernatants; lane A, fraction. The purified rhIL-15 were indicated with arrows. (c) rhIL-15 was purified by Ni^{+}-NTA column, separated on SDS-PAGE under native and denaturing condition, and stained with Coomassie blue R250.

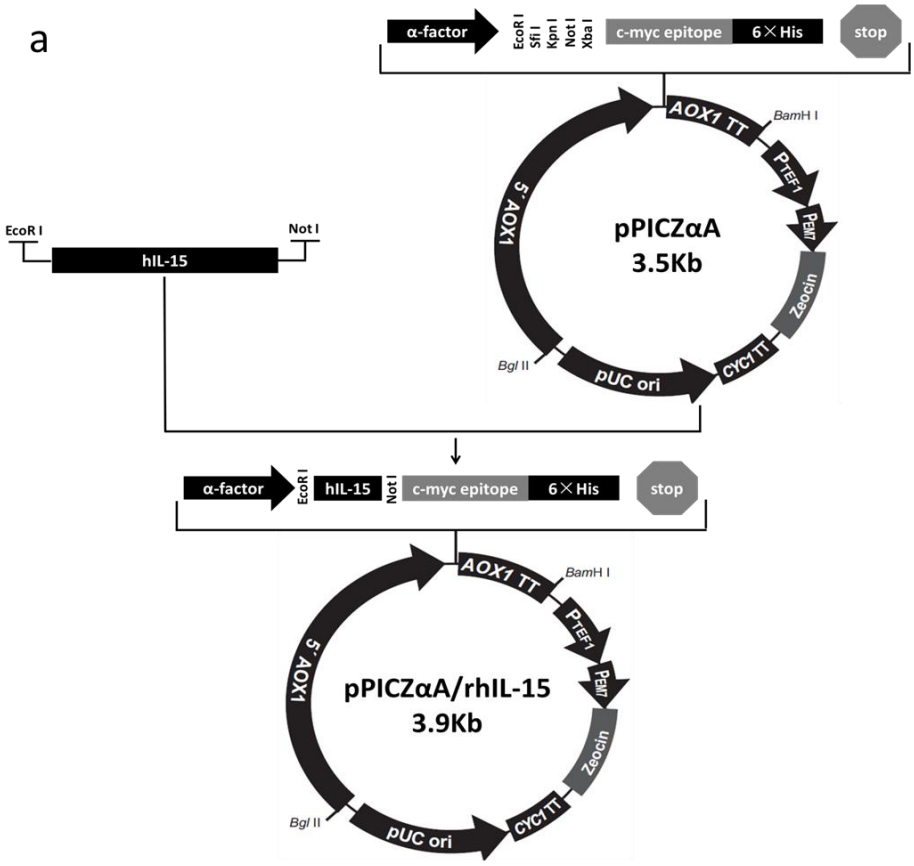
Fig.4 SDS-PAGE analysis of deglycosylated rhIL-15. The purified rhIL-15 proteins were deglycosylated by PNGase F. Lane M, protein markers; lane 1#, glycosylated rhIL-15; lane 2#, deglycosylated rhIL-15 by PNGase F; lane 3#; PNGase F (about 36 KDa). The bands indicated with arrows were cut and further analyzed by MALDI-TOF-TOF mass spectrometry.

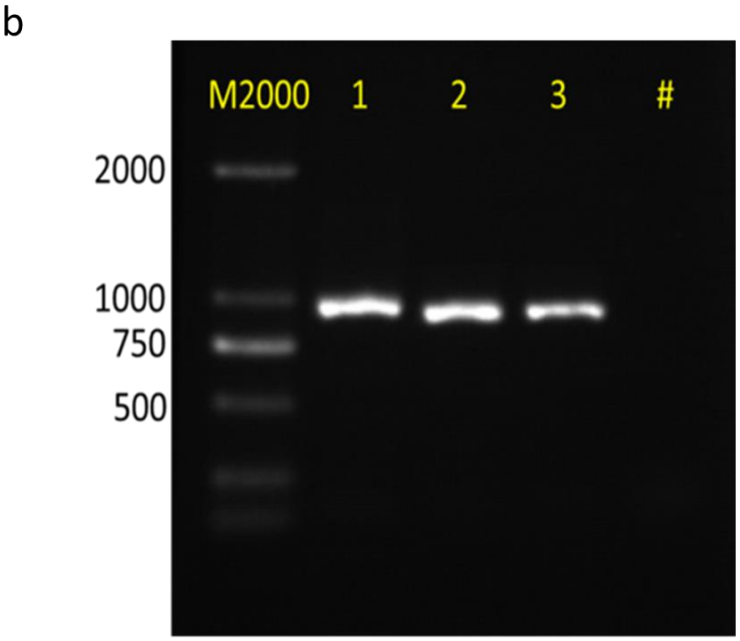
Fig.5 MALDI-TOF-TOF analysis of the purified deglycosylated rhIL-15. (a) Lower molecular weight protein band. (b) Higher molecular weight protein band.

Fig.6 Bioactivity of purified rhIL-15. CTLL-2 cells were incubated with rhIL-15 samples (0ng/mL, 0.01ng/mL, 0.05ng/mL, 0.25ng/mL, 2.5ng/mL, 25ng/mL) for 48 h. Proliferation of CTLL-2 cells was determined using Cell Titer-Glo, luminescence was read in a Wallac Victor absorbance/luminescence reader. Data represent the mean ± SD of triplicate tests, bars show mean and standard deviation of three independent experiments.

Fig.7 (a) Purified rhIL-15 stimulates the proliferation of human NK cells in vitro. 1×10^{6} human NK cells were incubated with rhIL-15 (50ng/mL) for 8 days and the same volume of PBS was used as negative control. Cells were collected at day 4 and day 8, stained with APC-CD56 and FITC-CD3, and analyzed on a Beckman-Coulter FC500 flow cytometer for the proportion of the cultures comprised of live NK cells. (b) Purified rhIL-15 stimulates the proliferation of human NK cells of peripheral blood, spleen and bone marrow in NSI mice. 3×10^{6} human NK cells were injected into each mouse and mice were treated intraperitoneally with 200ug IL-15 or 200ul PBS (n=3) once per day for 4 days, and on day 7, mice were sacrificed to detect NK cells in the peripheral blood, spleen and bone marrow. (c) The percentage of $CD45^{+}CD56^{+}$ NK cells of peripheral blood, spleen and bone marrow in rhIL-15 or PBS treated NSI mice(n=3). Error bars represent SEM; *P<0.05.

Figure.1.





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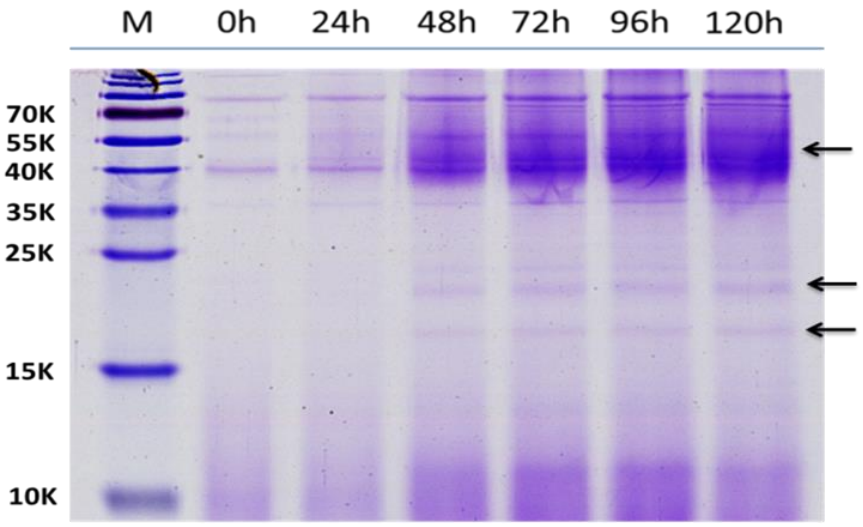

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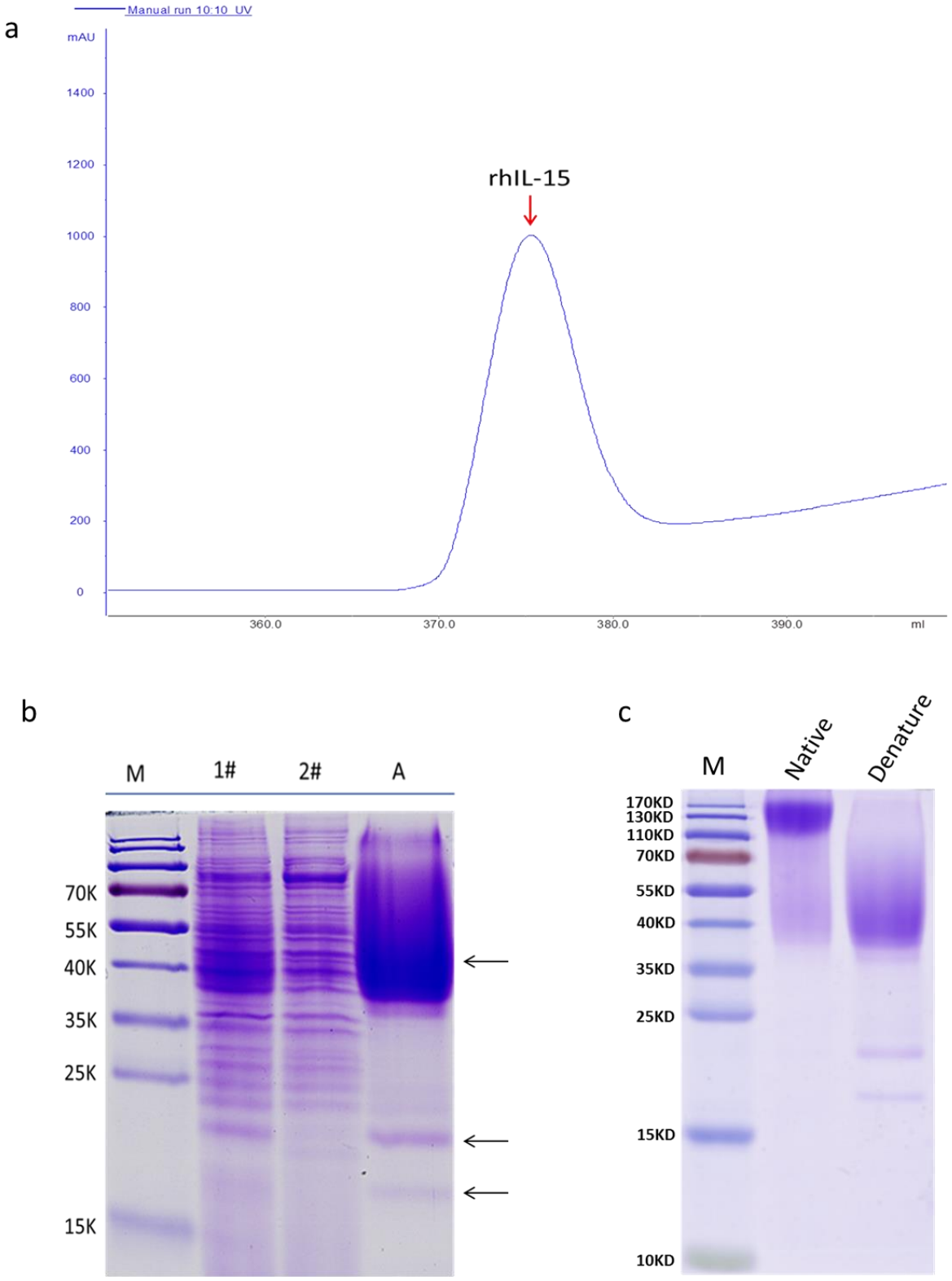

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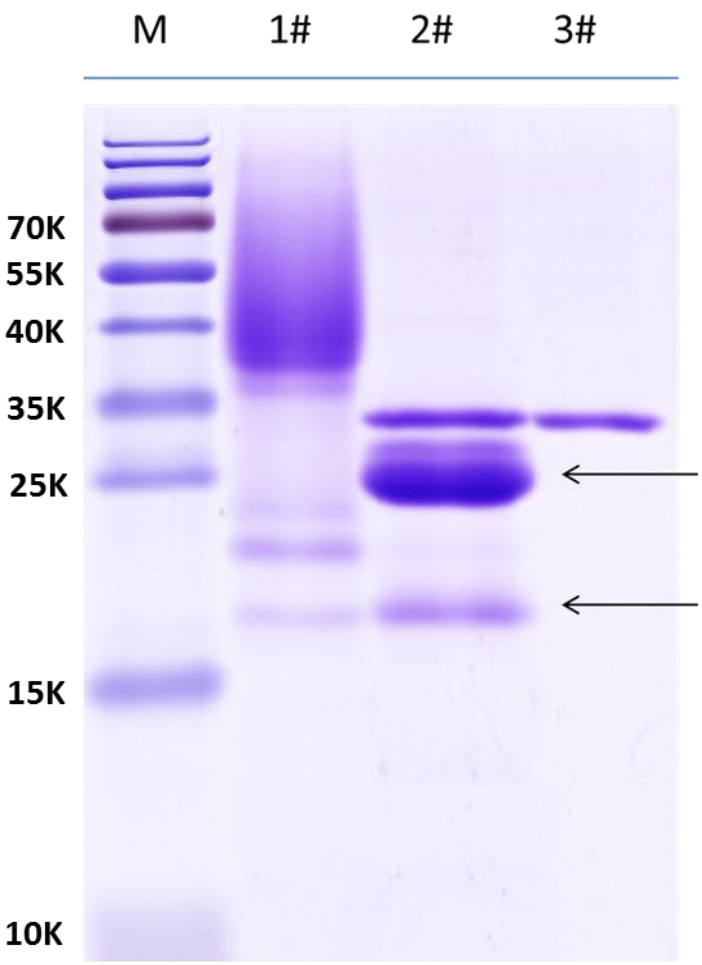
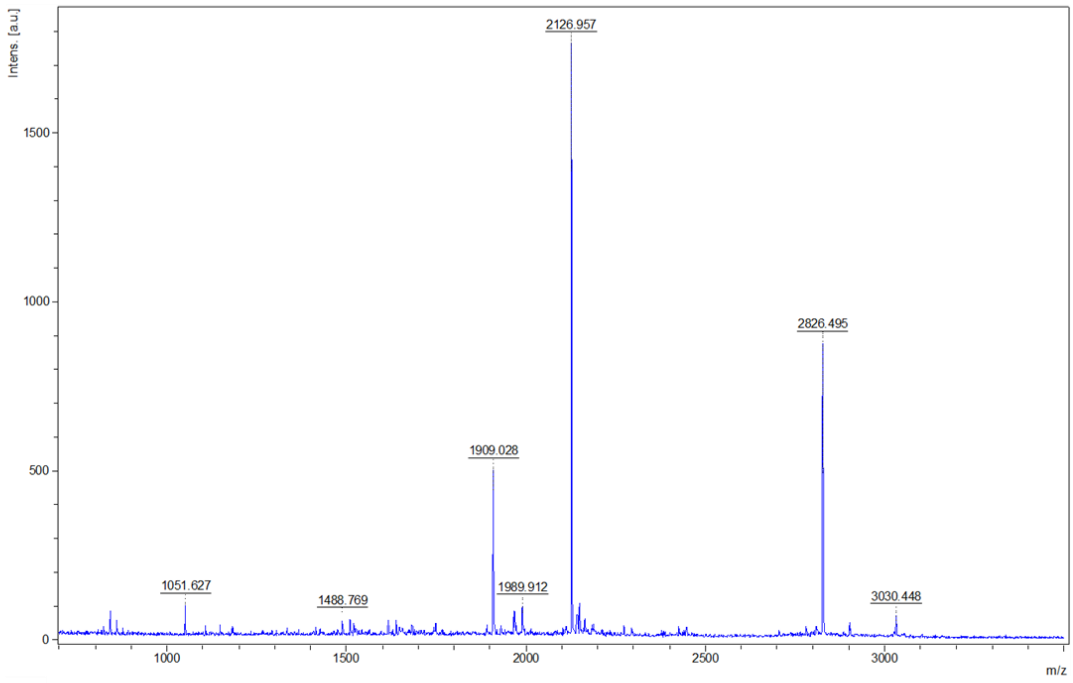


Figure.5.

a



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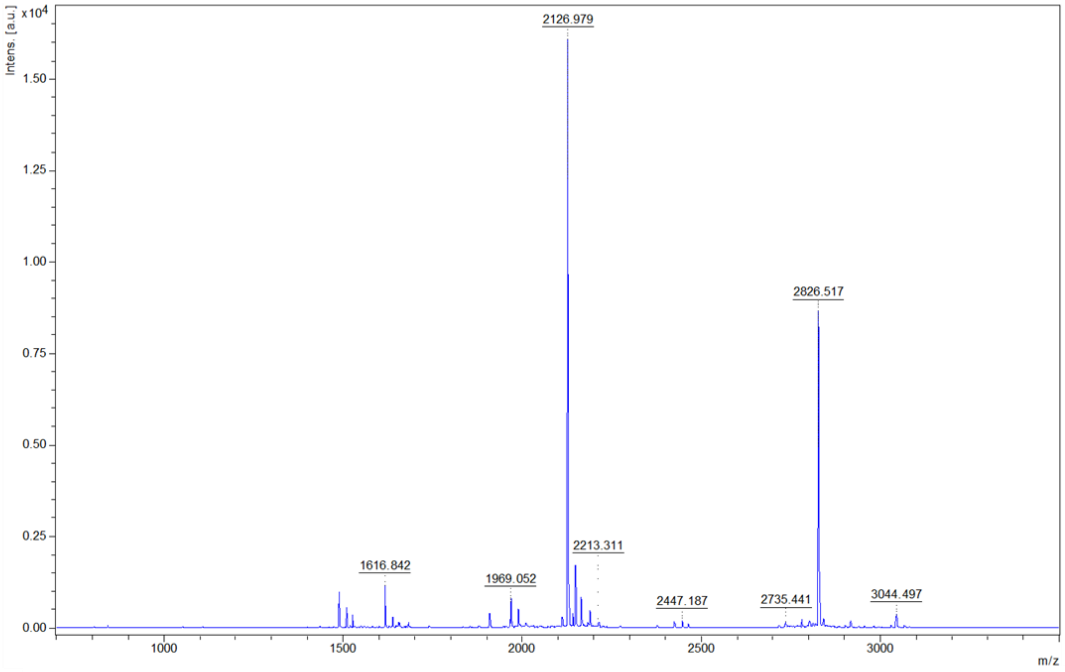

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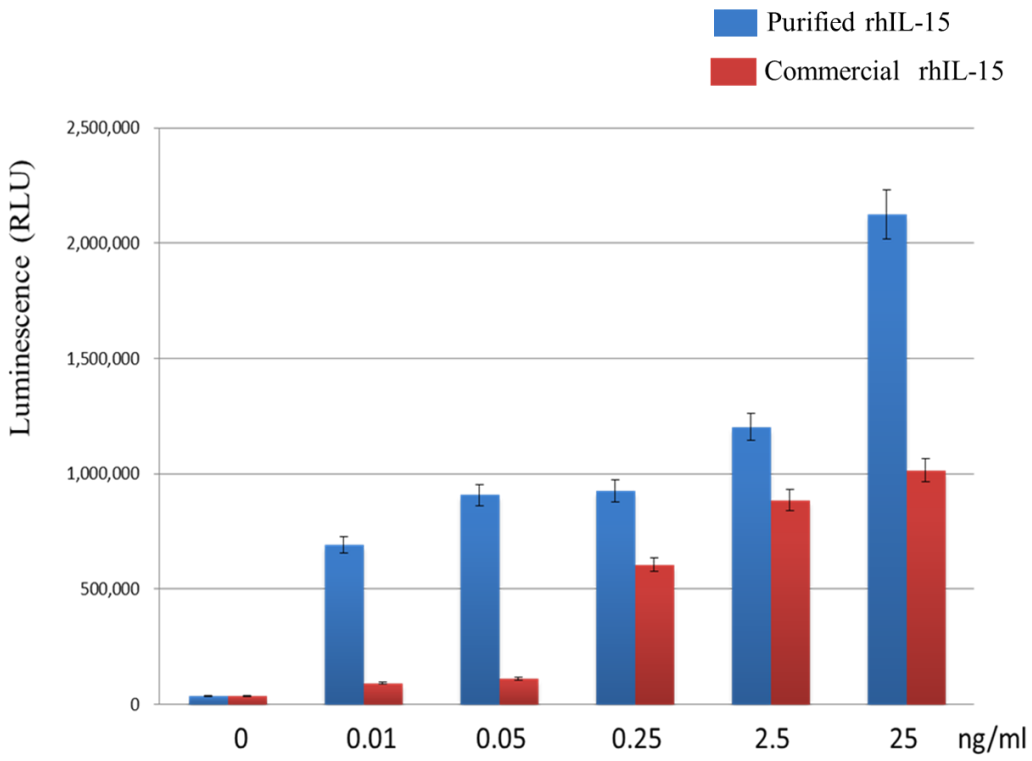
Figure.6.



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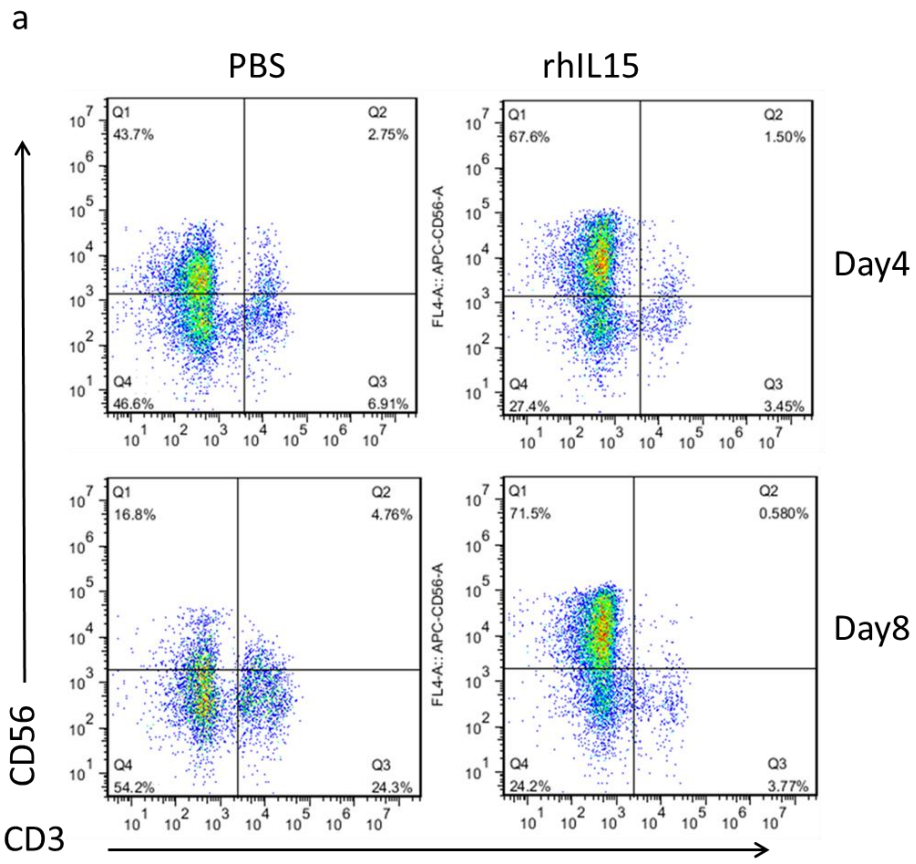

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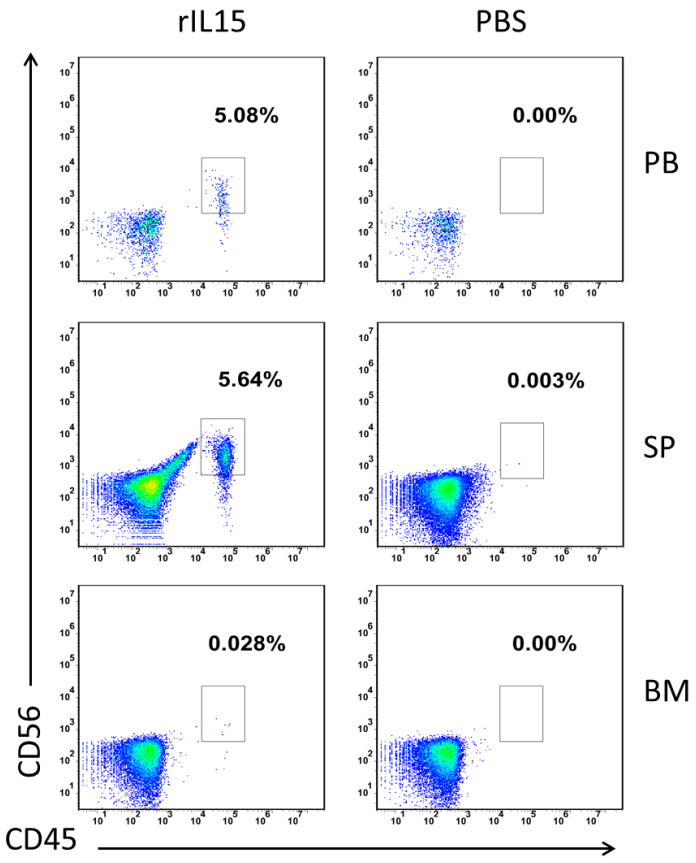

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